Phosphate-selective porins from the outer membranes of fluorescent *Pseudomonas* sp.

KEITH POOLE, THOMAS R. PARR, JR., AND ROBERT E. W. HANCOCK
Department of Microbiology, University of British Columbia, Vancouver, B.C., Canada V6T 1W5

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Phosphate starvation induced oligomeric proteins from the outer membranes of *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas aureofaciens*, and *Pseudomonas chlororaphis* were purified to homogeneity. The incorporation of the purified proteins into planar lipid bilayer membranes resulted in stepwise increases in membrane conductance. Single channel conductance experiments demonstrated that these proteins were all capable of forming small channels, similar to the *Pseudomonas aeruginosa* phosphate porin protein P, with average single channel conductances in 1 *M* KCl of between 233 and 252 pS. Single channel conductance measurements made in salts of varying cation or anion size indicated that the channels were uniformly anion selective. The measurement of single channel conductance as a function of KCl concentration revealed that all channels saturated at higher salt concentrations, consistent with the presence of an anion-binding site in the channel. Apparent K_d values for Cl^- binding were calculated and shown to vary only twofold (180–297 m*M*) among all channels, including protein P channels. Phosphate competitively inhibited chloride conductance through these channels with apparent I_{50} values of between 0.59 and 2.5 m*M* phosphate at 40 m*M* Cl^- and between 9.7 and 27 m*M* phosphate at 1 *M* Cl^-. These data were consistent with the presence of a phosphate-binding site in the channels of these phosphate-regulated proteins. Furthermore, they indicated that these channels exhibit at least a 20- to 80-fold higher affinity for phosphate than for chloride.

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Des protéines oligomères, induites par privation de phosphates, ont été obtenues des membranes externes de *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas aureofaciens* et *Pseudomonas chlororaphis*, et purifiées jusqu'à l'homogénéité. L'incorporation des protéines purifiées dans la bicouche planaire lipidique des membranes s'est traduite par des augmentations graduelles de la conductance membranaire. Des expériences de conductance par canaux individuels ont démontré que ces protéines étaient toutes capables de former de petits canaux, semblables à ceux produits par la phosphate porin protéine P de *Pseudomonas aeruginosa*, avec des conductances moyennes par canal dans du KCl 1 *M* entre 233 et 252 pS. Des mesures de conductance par canaux individuels dans des sels dont les cations et les anions variaient en dimension ont indiqué que les canaux étaient sélectionnés uniformément en fonction des anions. La mesure de conductance d'un canal individuel comme une fonction de la concentration en KCl a révélé que tous les canaux étaient saturés aux plus hautes concentrations, ce qui est en accord avec la présente des sites de liaisons anioniques dans les canaux. Les valeurs K_d apparentes pour les liaisons avec Cl^- ont été calculées et s'avèrent ne varier que du double (180–297 m*M*) parmi tous les canaux, incluant ceux des protéines P. Les phosphates ont inhibé la conductance des chlorures de façon compétitive à travers ces canaux, avec des valeurs de I_{50} apparents entre 0,59 et 2,5 m*M* de phosphate à 40 m*M* de Cl^- et entre 9,7 et 27 m*M* de phosphate à 1 *M* de Cl^-. Ces résultats furent concordants avec la présence de liaisons phosphates dans les canaux des protéines régulées par ces phosphates. De plus, ils ont indiqué que ces canaux avaient une affinité plus élevée pour les phosphates que pour les chlorures, d'au moins de 20 à 80 fois.
[Traduit par la revue]

Introduction

Porin proteins from Gram-negative bacteria form water-filled diffusion channels through the outer membrane which mediate the transmembrane diffusion of hydrophilic molecules below a defined molecular weight cutoff (the exclusion limit) (Nikaido 1979, Benz 1984). Porins, including the phosphate starvation inducible PhoE protein, generally form large (1–2 nm) channels which exhibit only weak ion selectivity in black lipid bilayer membranes (Benz *et al.* 1983; Benz 1984). In contrast, protein P, a porin protein from *Pseudomonas aeruginosa*, forms small (0.6 nm), anion-specific channels in reconstituted lipid bilayer membranes (Hancock *et al.* 1982; Benz *et al.* 1983).

Protein P is derepressed in wild-type cells of *P. aeruginosa* under conditions of phosphate deficiency (Hancock *et al.* 1982). The anion specificity of its channels was demonstrated to be due to an anion-binding site, within the channel (Benz *et al.* 1983; Benz 1984) formed by positively charged lysine residues (Hancock *et al.* 1983). Mutational and physiological studies (Hancock *et al.* 1982; Poole and Hancock 1983, 1984) showed that protein P was coregulated with components of a high-affinity inorganic phosphate transport system in *P. aeruginosa*, suggesting a role for protein P in this transport system. The recent demonstrations of a phosphate-binding site in the protein P channel (Hancock and Benz 1986) and the altered kinetics of high-affinity phosphate uptake in a mutant deficient in protein P (Poole and Hancock 1986*b*) strongly support this hypothesis. Phosphate-regulated porins have been identified in other bacteria including members of the families *Enterobacteriaceae* and *Pseudomonadaceae* (Overbeeke and Lugtenberg 1980; Sterkenburg *et al.* 1984; Verhoef *et al.* 1984; Bauer *et al.* 1985; Poole and Hancock 1986*a*). Based on their monomer molecular weights and detergent solubilities, most of these phosphate starvation inducible proteins could be grouped with the *Escherichia coli* PhoE porin (Poole and Hancock 1986*a*). However, the phosphate starvation inducible porins of the fluorescent pseudomonads, including *P. aeruginosa* (protein P), *P. putida*, *P. fluorescens*, *P. aureofaciens*, and *P. chlororaphis*, formed a clearly distinct group on the basis of these properties. To determine whether these proteins also resembled protein P functionally, we purified them and characterized their channel-forming properties in planar lipid bilayer membranes. We report here that these proteins form protein P like channels in all cases.

Materials and methods

Bacterial strains and media

Pseudomonas aeruginosa (ATCC No. 19305), *Pseudomonas putida* (ATCC No. 12633), *Pseudomonas fluorescens* (ATCC No. 949), *Pseudomonas chlororaphis* (ATCC No. 9446), and *Pseudomonas aureofaciens* (ATCC No. 13985) were the strains employed in this study. With the exception of *P. fluorescens*, all strains were type strains.

The sodium Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffered phosphate-sufficient and phosphate-deficient minimal media have been described previously (Hancock *et al.* 1982). Strains were routinely maintained on L agar (1% (w/v) tryptone – 0.5% (w/v) yeast extract – 0.05% (w/v) NaCl – 2% (w/v) Bactoagar) and phosphate-sufficient minimal medium plates (phosphate-sufficient medium + 2% (w/v) Bactoagar).

Cell fractionation and polyacrylamide gel electrophoresis

Cell envelopes were prepared as described previously (Nicas and Hancock 1980). Solubilized cell envelopes were prepared by extracting once with 2% (w/v) Triton X-100 – 20 m*M* Tris–HCl, pH 8.0 – 10 m*M* ethylenediaminetetraacetate (EDTA), pH 8.0, after 30 min incubation at 37°C in the presence of 1 mg lysozyme/mL as described (Hancock *et al.* 1981). Sodium dodecyl sulphate (SDS) – polyacrylamide gel electrophoresis (PAGE) was performed as described previously (Hancock and Carey 1979) using a 12% (w/v) acrylamide running gel.

Preparation of a protein P – Affi-gel-10 column

Protein P, purified as described previously (Hancock *et al.* 1982), was passed across a Biogel P-10 column equilibrated with 0.1 *M* acetic acid – sodium acetate buffer, pH 5.0 – 0.1% (w/v) SDS. Peak fractions, measured by absorbance at 280 nm (A_{280}), were pooled and the protein (1.5 mg) was cross-linked to approximately 0.65 mL of Affi-gel-10 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) for 2 h at room temperature as recommended by the manufacturer. The column was stored at 4°C in phosphate-buffered saline (PBS), pH 7.4 (Mutharia and Hancock 1983) containing 0.1% (w/v) Triton X-100. Prior to use, the column was washed exhaustively with PBS to remove excess detergent.

Preparation of antisera and construction of an anti-protein P immunoadsorbent column

Antiserum specific for protein P trimers was prepared as described previously (Poole and Hancock 1986*b*). Protein P trimer specific antibodies were purified free of serum proteins by incubating the protein P trimer specific antiserum (200 μL) on the above protein P – Affi-gel-10 affinity column (1.8 × 0.7 cm). After incubation at room temperature for 45 min, the column was washed with 3 mL PBS, followed by 3 mL PBS + 0.25 M NaCl, to wash off unbound and nonspecifically bound material, respectively. Bound antibodies were eluted with 3 mL 0.1 *M* glycine–HCl, pH 2.5, and fractions (300 μL) collected in tubes containing enough solid Tris base (Schwartz-Mann, Cambridge, MA) to increase the pH of the fractions to between 7 and 9. The fractions containing antibody to protein P, as measured by an enzyme-linked immunosorbent assay (ELISA) (Mutharia and Hancock 1985), were pooled and dialysed against 1 L of 0.1 *M* borate buffer, pH 9.0 (0.1 *M* boric acid, pH 9.0 – 0.1 *M* KCl) for 24 h at 4°C, with one buffer change. The dialysed antibodies (2.3 mL at an absorbance at 280 nm of 0.22) were then cross-linked to 0.5 gm CNBr-activated Sepharose 4B (Pharmacia, Upsalla, Sweden) in 0.1 *M* borate buffer, pH 9.0, for 16 h at 4°C as recommended by the manufacturer. The final column volume was approximately 1.0 mL. The column was stored at 4°C in PBS.

Purification of phosphate starvation induced outer membrane proteins

Two methods were used for the purification of phosphate starvation induced outer membrane proteins. The first was an affinity chromatography method utilizing an immunoadsorbent column. Triton X-100 insoluble cell envelopes prepared from 100 mL stationary phase cultures of phosphate-limited cells were solubilized in 1 mL 2% (w/v) Triton X-100 – 20 mM Tris–HCl, pH 8.0 – 10 m*M* EDTA containing 1 mg lysozyme/ mL at 37°C for 30 min. The Triton X-100 – EDTA – lysozyme soluble fractions (300 μL) were subsequently incubated on the anti-protein P immunoadsorbent column at room temperature. After 45 min incubation, the column was washed successively with 3 mL of 2% Triton X-100 – 20 m*M* Tris–HCl, pH 8.0, and 3 mL of 2% (w/v) Triton X-100 – 20 mM Tris-HCl, pH 8.0 – 0.5 *M* NaCl, to remove unbound and nonspecifically bound material, respectively. Material bound specifically to the column was eluted with 3 mL of 1% (w/v) Triton X-100 – 0.1 *M* glycine–HCl, pH 2.5, and fractions collected in tubes containing solid Tris base as described above. Peak fractions, as determined by SDS–PAGE, were pooled.

The second method involved electroelution of proteins from SDS– polyacrylamide gels. Triton X-100 – EDTA – lysozyme soluble cell envelopes (described above) (300 μL) were solubilized at room temperature in an equal volume of solubilization mix without 2-mercaptoethanol (Hancock and Carey 1979), loaded onto individual SDS–polyacrylamide slab gels (1.5 mm thickness), and electrophoresed to separate protein components. Phosphate starvation induced membrane protein oligomer bands were subsequently excised and the proteins extracted from the gel via electroelution as described previously (Parr *et al.* 1986). The eluted proteins (5 mL, final volume) were concentrated 10-fold against solid polyethylene glycol (20 000 molecular weight, Sigma Chemical Co., St. Louis, MO, U.S.A.) before being dialysed at room temperature for 16 h against 1 L of 0.1% (w/v) Triton X-100 – 20 m*M* Tris–HCl, pH 8.0, with one buffer change.

Black lipid bilayer experiments

The methods used for black lipid bilayer experiments have been described previously in detail (Benz *et al.* 1979; Benz and Hancock 1981). The apparatus consisted of a Teflon chamber with two compartments separated by a small hole (0.1 mm^2 for single channel experiments; 2 mm^2 for macroscopic conductance experiments). A membrane was formed across the hole by painting on a solution of 1.5% (w/v) oxidized cholesterol in *n*-decane. Bilayer formation was indicated by the membrane turning optically black to incident light. In single channel conductance experiments, conductance through the pores was measured after application of a given voltage using a pair of Ag/AgCl electrodes inserted into the aqueous solutions on both sides of the membrane. The current through the pores was boosted by a preamplifier, monitored by a storage oscilloscope, and recorded on a strip chart recorder.

The procedure for macroscopic conductance inhibition experiments has been described (Hancock and Benz 1986). Briefly, the Ag/AgCl electrodes were replaced with Calomel electrodes and the current through the pores was monitored with a Keithley 610B electrometer. After addition of purified protein P to the solutions bathing the lipid bilayer membrane, the increase in conductance (measured as current increase) was followed for 15–25 min or until the rate of increase had slowed considerably. At this time membrane conductance had increased 2–4 orders of magnitude. The bathing solutions in both compartments were stirred gently (approximately 60 revolutions per minute) with a magnetic stir bar and aliquots of phosphate added to both compartments. Sufficient time (30– 90 s) was allowed for the new current level to be established before addition of subsequent aliquots.

Results

Purification of the phosphate starvation inducible outer membrane proteins of the fluorescent pseudomonads

Members of the *Pseudomonas fluorescens* branch of the family *Pseudomonadaceae*, including *P. putida*, *P. fluorescens*, *P. aureofaciens*, and *P. chlororaphis*, were previously demonstrated to synthesize oligomeric, heat-modifiable outer membrane proteins which exhibited a number of properties in common with protein P of *P. aeruginosa* (Poole and Hancock 1986*a*). Using the observed immunological cross-reactivity of these proteins with protein P, an attempt was made to purify these phosphate-regulated proteins by specific retention on an

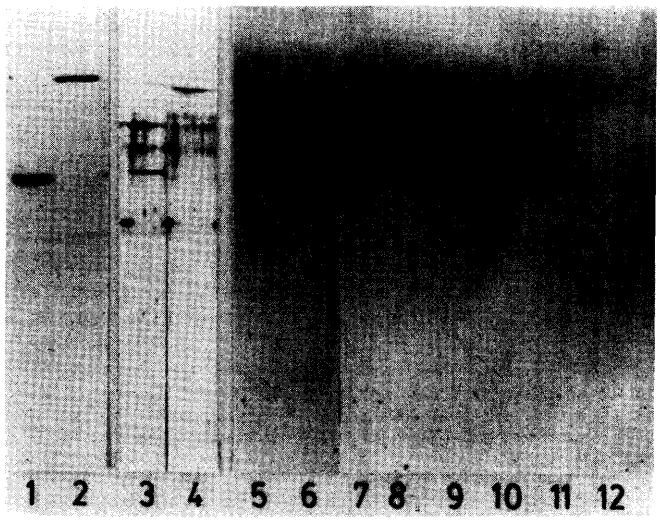

FIG. 1. SDS–polyacrylamide gel electrophoretogram of purified phosphate starvation inducible outer membrane proteins of the fluorescent pseudomonads. Phosphate starvation inducible proteins were purified from the outer membranes of the following: lanes 1, 2, 11, and 12, *P. aeruginosa* (i.e., protein P); lanes 3 and 4, *P. fluorescens*; lanes 5 and 6, *P. putida*; lanes 7 and 8, *P. aureofaciens*; lanes 9 and 10, *P. chlororaphis*. The proteins in lanes 3, 4, 11, and 12 were purified via affinity chromatography using a rabbit anti-protein P immunoadsorbent column. The proteins in lanes 1, 2, and 5–10 were purified by electroelution from polyacrylamide gels. Samples were solubilized prior to electrophoresis at 88°C (lanes 1, 3, 5, 7, 9, 11), resulting in a monomer band of molecular weight 48 000, or at 23°C (lanes 2, 4, 6, 8, 10, 12) resulting in a trimer band of apparent molecular weight 90 000. The methods for identifying these bands and the protein standards used for molecular weight estimations were described previously (Hancock *et al.* 1982). Lanes 3 and 4 were stained for protein using a sensitive silver staining procedure (Wray *et al.* 1981). Artifact bands visible in these lanes are a product of the silver staining procedure. All other lanes were stained by Coomassie brilliant blue. The faint continuous band seen in the middle of the gel is an artifact and was observable in lanes where no protein was loaded.

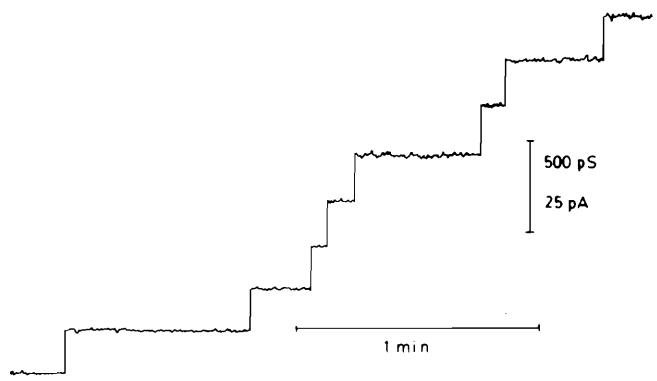

FIG. 2. Strip chart recordings of stepwise increases in the conductance of a small (0.1 mm^2) oxidized cholesterol membrane (1.5% in *n*-decane) caused by the addition of 10 ng/mL of the phosphate starvation induced outer membrane protein from *P. putida*.

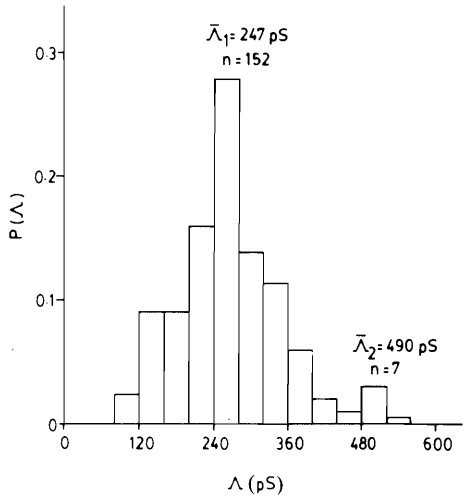

FIG. 3. Histogram of the conductance fluctuations observed with membranes of oxidized cholesterol (1.5% in *n*-decane) in the presence of the phosphate starvation induced outer membrane protein of *P. putida* and 1 *M* KCl, pH 6.0, in the aqueous phase. The applied voltage was 50 mV and the temperature was 25°C. $P(\Lambda)$ is the probability of a given conductance increment Λ (taken from recorder tracings such as that shown in Fig. 2).

immunoadsorbent column constructed with the protein P trimer specific antiserum described previously (Poole and Hancock 1986*b*). Protein P, the original antigen, was readily purified in reasonable quantities by this method (Fig. 1, lanes 1 and 2). It was also possible to isolate the other phosphate starvation inducible outer membrane proteins using this column, although the yields were substantially lower, requiring a sensitive silver staining procedure to detect the proteins on SDS–polyacrylamide gels (e.g., Fig. 1, lanes 3 and 4). Apparently, the cross-reactive antibodies in the protein P trimer specific antiserum represent only a minor or low-affinity component of this antiserum. The purified proteins occurred as higher molecular weight oligomers on SDS–polyacrylamide gels when solubilized at room temperature prior to electrophoresis (e.g., Fig. 1, lane 4), dissociating to lower molecular weight monomers when solubilized at 88°C (e.g., Fig. 1, lane 3). This was consistent with the observed properties of these proteins in phosphate-limited cell envelope preparations (not shown) and with the properties of purified protein P (Fig. 1, lanes 1 and 2).

To improve yields, these proteins were also purified using a procedure for the electroelution of proteins out of SDS–polyacrylamide gels (Parr *et al.* 1986). Phosphate starvation induced protein containing extracts prepared from each of the above strains were run on SDS–polyacrylamide gels, the relevant protein oligomer bands excised, and the protein was electroeluted from the gel. This method produced substantially increased yields of all proteins (Fig. 1, lanes 5–12) which were easily visible on Coomassie stained gels. Again, the purified proteins retained their oligomeric structure, as attested by their resistance to SDS denaturation (Fig. 1, lanes 6, 8, 10, and 12), unless heated at high temperature (Fig. 1, lanes 5, 7, 9, and 11).

Single channel experiments

When the purified phosphate starvation inducible outer membrane proteins were added in small quantities (5–10 ng/mL) to the aqueous solutions bathing a black lipid bilayer membrane, membrane conductance was seen to increase in a stepwise fashion (e.g., Fig. 2), presumably due to the incorporation of individual protein oligomers into the membrane, as suggested for other porins (Benz *et al.* 1979; Benz and Hancock 1981). Most of the observed single channel conductance increments were distributed about a mean (e.g., Fig. 3, Λ_1), although larger increments were also seen at 2 (Fig. 3, Λ_2), 3 and 4 (not shown) times the average single channel conductance. These larger increments probably represented aggregates

TABLE 1. Channel-forming properties of affinity-purified and electroeluted phosphate starvation inducible outer membrane oligomers of the fluorescent pseudomonads

Source of phosphate starvation inducible outer membrane protein	Affinity purified		Electroeluted	
	Single channel conductance (pS)[a]	n[b]	Single channel conductance (pS)	n
P. aeruginosa	239	317	234	224
P. putida	233	74	247	307
P. fluorescens	241	117	nd	nd
P. aureofaciens	237	54	252	198
P. chlororaphis	243	45	237	201

[a]Average value for n events. The probability histograms of single channel conductances (Fig. 3) were overlapping for all proteins.
[b]Number of single channel events measured.

TABLE 2. Single channel conductance of phosphate starvation inducible porin proteins of the fluorescent pseudomonads in salts of varying anion and cation size

Source of porin	Average single channel[a] conductance (pS) in:		
	K^+Cl^-	$Tris^+Cl^-$	K^+Hepes^-
P. aeruginosa	158	141	—[b]
P. putida	144	143	—
P. fluorescens	156	149	—
P. aureofaciens	164	169	—
P. chlororaphis	166	167	—

[a]Average of at least 60 single channel events. Salts were employed at a concentration of 0.5 *M* at pH 6.0. Ion radii (in nm) are as follows: K^+, 0.133; Cl^-, 0.181; $Tris^+$, 0.670. $Hepes^-$, an ellipsoid molecule, has the dimensions 1.4 × 0.6 × 0.5 nm.
[b]No conductance increments were resolved. The minimum resolvable conductance increment in the above experiment was 10 pS, although the single channel conductance of protein P channels in K^+Hepes^- was previously found to be <2 pS (Hancock *et al.* 1982).

or simultaneous insertions of two or more protein oligomers into the bilayer membrane (Hancock *et al.* 1982). The average single channel conductances in 1 *M* KCl, measured for a given protein purified by either affinity chromatography or electroelution, were very similar (Table 1), confirming both the utility of the anti-protein P immunoadsorbent column in purifying functional cross-reactive molecules and the general applicability of the electroelution procedure in purifying functionally active porin proteins. The average single channel conductance values obtained for the individual phosphate starvation inducible proteins were also very similar to one another, falling between 233 and 252 pS (Table 1). These values were substantially less than those obtained for the phosphate starvation inducible PhoE porins of *E. coli* and *Salmonella typhimurium* (approximately 2 nS (Benz *et al.* 1983; Bauer *et al.* 1985)). They were, however, in excellent agreement with the observed single channel conductance of protein P in 1 *M* KCl (Table 1).

Ion selectivity

To probe the ion selectivity of these channels, single channel conductance was measured in the presence of salts of varying cation or anion size. The anion-specific protein P channel has been previously demonstrated to yield average single channel conductances, the magnitudes of which were dependent exclusively upon the size of the anion (Benz *et al.* 1983). Thus,

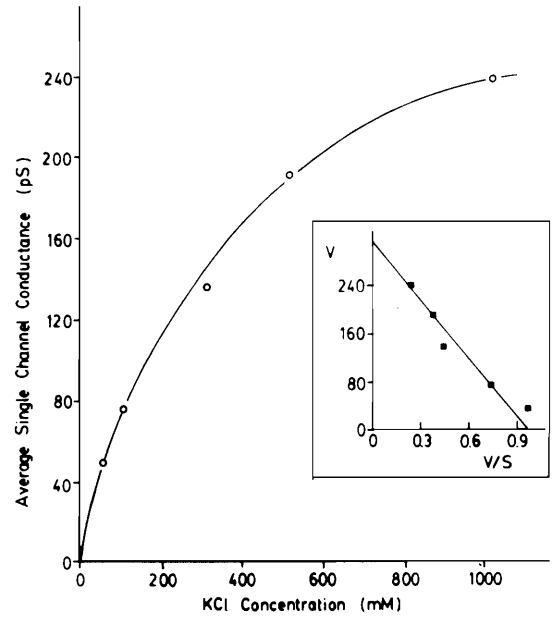

FIG. 4. Average single channel conductance of the phosphate starvation induced porin protein of *P. aureofaciens* as a function of the KCl concentration in the aqueous solution bathing an oxidized cholesterol (1.5% in *n*-decane) membrane. The applied voltage was 50 mV and the temperature was 25°C. The aqueous phase contained approximately 10 ng/mL of porin protein at KCl concentrations of 300 m*M* and higher. For KCl concentrations below 300 m*M*, 100 ng/mL of protein had to be added to obtain a sufficient number of single channels. INSET: An Eadie–Hofstee plot of the data obtained from measurements of single channel conductance (V) as a function of the KCl concentration (S). Binding constants (Table 3) were obtained from such a plot using least squares analysis.

conductance through protein P channels was demonstrated to be inversely related to the size of the anion (Benz *et al.* 1983), while remaining basically unaffected by changes in cation size of the salt bathing a protein P containing lipid bilayer membrane (Hancock *et al.* 1982). The results in Table 2 demonstrated that in all cases the observed single channel conductance was dependent upon anion size only, such that increasing the anion size in the case of K^+Hepes^- (anion dimensions of 1.4 × 0.6 × 0.5 nm compared with an anion radius of 0.181 nm for K^+Cl^-) resulted in no detectable conductance increments, while increasing the cation size in the case of $Tris^+Cl^-$ (cation radius of 0.67 nm compared with 0.133 nm for K^+Cl^-) yielded a single channel conductance which was not substantially different from that observed in K^+Cl^-. These data were consistent with the formation of anion-selective, if not specific, channels by these proteins.

The anion specificity of protein P has been shown to be due to the presence of an anion-binding site within the channel (Benz *et al.* 1983). Thus conductance through protein P channels saturates at higher salt concentrations (Benz *et al.* 1983), in contrast to other porins, including PhoE, which lack binding sites and typically reveal a linear dependence of single channel conductance on salt concentration (Benz and Hancock 1981; Benz *et al.* 1984). To determine if the anion selectivity of the phosphate-regulated porin proteins could be attributed to binding sites within their respective channels, single channel conductance was measured as a function of salt (KCl) concentration. In every case, single channel conductance was seen to saturate at higher salt concentrations (e.g., Fig. 4) consistent with the presence of a binding site within these channels. By

TABLE 3. Binding affinities for chloride and orthophosphate of the phosphate starvation inducible porin proteins of the fluorescent pseudomonads

Source of porin	K_d for Cl^- (mM)[a]	I_{50} for phosphate (mM)[b] at: 40 mM Cl^-	1 M Cl^-
P. aeruginosa	153	0.59	12.7
P. putida	192	1.08	nd
P. fluorescens	220	nd[c]	9.7
P. aureofaciens	297	nd[c]	27.0
P. chlororaphis	204	2.40	nd

[a]The average single channel conductance from at least 75 recorded events was determined at each of five concentrations of KCl between 50 and 1000 mM. The data was plotted as an Eadie–Hofstee plot (Fig. 4, inset) from which K_d values were obtained by least squares analysis.

[b]Inhibition of macroscopic chloride conductance by phosphate was carried out as described in Materials and methods. The percent inhibition of initial conductance was measured for different concentrations of phosphate and the data plotted as an Eadie–Hofstee plot (see Fig. 4, inset) from which average I_{50} values (defined as the concentration of phosphate yielding 50% inhibition of chloride conductance) were obtained by least squares analysis of three independent experiments.

[c]It has been demonstrated, for certain porin proteins, that the rate of porin incorporation into black lipid bilayer membranes decreases drastically at low concentrations of salt in the aqueous phase (R. E. W. Hancock, unpublished observation). At the levels of porin protein available, the numbers of porin channels inserting into the membrane at 40 mM KCl were insufficient to permit the accurate measurement of phosphate inhibition of chloride conductance, which usually required approximately 1000 channels per membrane (see Materials and methods).

replotting the data as an Eadie–Hofstee plot (e.g., Fig. 4, inset), K_d values for Cl^- binding were derived (Table 3). While some variability in the affinity of Cl^- for each of the channels was observed, there was only a twofold range in K_d values of Cl^- binding for all channels, including protein P.

Phosphate inhibition of macroscopic conductance

The presence of a phosphate-binding site within *P. aeruginosa* protein P channels has been previously confirmed by the ability of orthophosphate to inhibit Cl^- conductance through protein P channels (Hancock and Benz 1986). To identify potential phosphate-binding sites in the phosphate starvation induced porin proteins of *P. putida*, *P. fluorescens*, *P. aureofaciens*, and *P. chlororaphis*, this strategy was also applied in this study. Thus after formation of a lipid bilayer, a small amount of protein was added to the aqueous solution bathing the lipid membrane and conductance followed until the rate of increase had slowed considerably (usually 15–25 min). At this time membrane conductance had usually increased by 2–4 orders of magnitude and >1000 channels were present in the membrane. Aliquots of phosphate were added sequentially and the new conductance level measured after each addition.

For each protein studied, phosphate addition was seen to decrease the level of conductance originally observed in the presence of KCl alone, and the magnitude of this decrease was directly related to the concentration of phosphate added (e.g., Fig. 5). By plotting the data as percent inhibition of chloride conductance as a function of percent inhibition of chloride conductance – phosphate concentration (i.e., an Eadie–Hofstee plot) (Fig. 5, inset), it was possible to derive an apparent I_{50} value for phosphate inhibition of chloride conductance for each of the phosphate-regulated porins (Table 3). These data were consistent with the presence of a phosphate-binding site within each of these channels. The apparent I_{50} values varied with the concentration of KCl, ranging from 9.7 to 27 mM phosphate in 1 M KCl, and from 0.59 to 2.5 mM phosphate in 40 mM KCl (at pH 7) (Table 3). At a given concentration of KCl, however, the variation in I_{50} values obtained for all of the channels did not exceed fourfold, indicating that the relative affinities of these channels for phosphate were quite similar.

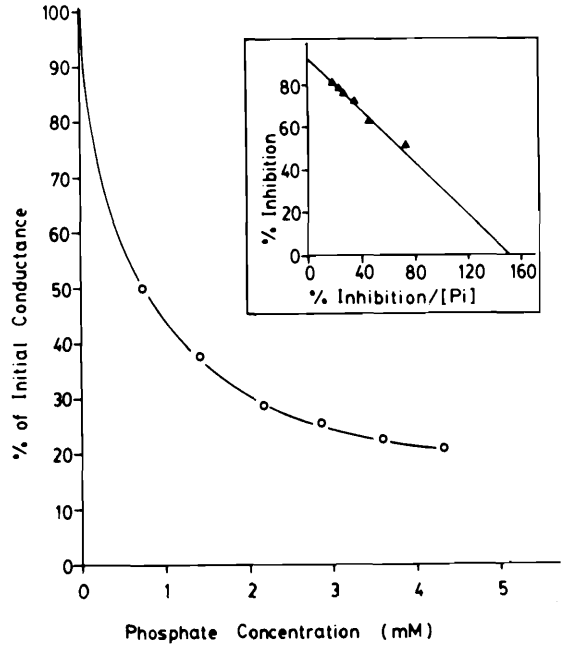

FIG. 5. Phosphate inhibition of chloride (Cl^-) flux through protein P channels. Protein P (100 ng/mL) was added to the aqueous solution (40 mM KCl – 1 mM Tris–KCl, pH 7.0) bathing an oxidized cholesterol (1.5% in *n*-decane) membrane and the membrane conductance allowed to increase until it had stabilized (usually at a level 2 to 4 orders of magnitude higher than the initial level). At this time aliquots of potassium phosphate buffer, pH 7.0, were added to the aqueous phase on both sides of the membrane and the new conductance level was recorded after each addition. The percent of initial Cl^- conductance was calculated and plotted as a function of the aqueous phase phosphate concentration. The applied voltage was 20 mV and the temperature was 25°C. INSET: An Eadie–Hofstee plot of the data derived from measurements of the percent inhibition of chloride conductance as a function of phosphate concentration [P_i]. I_{50} values for phosphate inhibition of chloride conductance (Table 3) were calculated using least squares analysis.

Discussion

It was previously suggested (Poole and Hancock 1986*a*) that phosphate starvation inducible outer membrane proteins expressed by members of the families *Pseudomonadaceae* and *Enterobacteriaceae* could be grouped into two classes based on monomer molecular weights and the detergent conditions necessary to release the proteins from the peptidoglycan. The best-studied representatives of these two classes are the *E. coli* PhoE protein and *P. aeruginosa* protein P, respectively. These two porins are also distinguishable from a functional view point. Thus, while the PhoE protein forms large (1 nm), weakly anion selective channels (Darveau *et al.* 1984; Benz *et al.* 1985) which lack strong binding sites for anions or phosphate (Benz *et al.* 1984), protein P channels are small (0.6 nm) (Hancock *et al.* 1982), and possess binding sites for anions and phosphate (Hancock and Benz 1986; Table 3) consistent with the observed anion specificity and phosphate selectivity of this channel (Hancock and Benz 1986; Table 3). The demonstration here that those proteins resembling protein P in molecular weight and detergent solubility also formed anion-specific – phosphate-

selective channels similar to that of protein P (Tables 2 and 3) supports the existence of these two classes, as does the observation that those enterobacterial phosphate-regulated porin proteins which have been examined (Verhoef *et al.* 1984; Bauer *et al.* 1985) form larger, less selective, PhoE-like channels.

Based on the results of DNA and rRNA hybridization studies, the strains examined here have been classified in the same homology group (the *Pseudomonas fluorescens* group) and are clearly distinct from other species of the family *Pseudomonadaceae* (Palleroni *et al.* 1973). Despite differences in the biochemical properties of those strains which produce a protein P like, phosphate starvation inducible porin, they appear to have additional, quite similar, outer membrane proteins. Each of these fluorescent pseudomonads produces a lipoprotein which cross-reacts immunologically with outer membrane protein H2 of *P. aeruginosa* PA01 (Mutharia and Hancock 1985). In addition, all of these strains produce a major, constitutive outer membrane protein, which resembles porin protein F both in molecular weight and inability to form SDS-stable oligomers in polyacrylamide gels (Mutharia and Hancock 1983; K. Poole, unpublished data). In contrast, members of the *Enterobacteriaceae* and other pseudomonads, including *P. cepacia* and *P. pseudomallei*, each produce a major, constitutive outer membrane protein which forms native oligomers which are resistant to denaturation by SDS at temperatures below 60°C (Lugtenberg and van Alphen 1983; K. Poole, unpublished data). Thus, these data are consistent with the existence of an evolutionary–taxonomic link between the fluorescent *Pseudomonas* sp. and emphasize the potential importance of outer membrane protein profiles and antigenic similarities amongst surface proteins as indicators of such links.

The existence of a protein P like porin in a number of distinct bacterial species affords the opportunity to perform comparative studies on the biogenesis of these proteins and to identify their functional domains. Given the similarities of the functional properties of these proteins, which were quite distinct from other phosphate-regulated porins, it should be possible to identify regions of the protein involved in, for example, anion–phosphate selectivity as regions of close homology in the genes encoding these proteins. Such data will undoubtedly contribute to our goal elucidating the topology of protein P in *P. aeruginosa*.

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